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Tremorgenic and neurotoxic paspaline-derived indole-diterpenes: biosynthetic diversity, threats and applications

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Abstract

Indole-diterpenes (IDTs) such as the aflatrems, janthitrems, lolitrems, paspalitrems, penitrems, shearinines, sulpinines, and terpendoles are biogenetically related but structurally varied tremorgenic and neurotoxic mycotoxins produced by fungi. All these metabolites derive from the biosynthetic intermediate paspaline, a frequently occurring IDT on its own right. In this comprehensive review, we highlight the similarities and differences of the IDT biosynthetic pathways that lead to the generation of the main paspaline-derived IDT subgroups. We survey the taxonomic distribution and the regulation of IDT production in various fungi and compare the organization of the known IDT biosynthetic gene clusters. A detailed assessment of the highly diverse biological activities of these mycotoxins leads us to emphasize the significant losses that paspaline-derived IDTs cause in agriculture, and compels us to warn about the various hazards they represent towards human and livestock health. Conversely, we also describe the potential utility of these versatile molecules as lead compounds for pharmaceutical drug discovery, and examine the prospects for their industrial scale manufacture in genetically manipulated IDT producers or domesticated host microorganisms in synthetic biological production systems.

Keywords

Indole-diterpene; fungal secondary metabolite; biosynthesis; mycotoxin; food and feed safety; drug discovery; heterologous production

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Conflict of interest

I. P. declares no conflict of interests. I. M. has disclosed financial interests in Teva Pharmaceuticals Works Ltd., Hungary and DSM Nutritional Products, LLC, USA which are unrelated to the subject of the research presented here. L. K., Z. S., and L. T. are employees of Teva Pharmaceutical Works Ltd., Hungary. Responsibility for the conclusions drawn, and the opinions expressed in this article are solely those of the authors and are not shared by Teva Pharmaceutical Works Ltd.

Compliance with ethical standards

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the Authors.

Introduction

Indole-diterpenes (IDTs) are small molecule secondary metabolite natural products biosynthesized by a select group of ascomycetous fungi including *Aspergillus, Penicillium, Emericella, Eupenicillium, Claviceps, Epichloë, Escovopsis*, *Neotyphodium, Periglandula* and *Tolypocladium* species, as well as the zygomycetous fungus *Mucor irregularis* (Saikia et al. 2008; Schardl et al. 2013; Gao et al. 2016; Dhodary et al. 2018; Supplementary Table S1). IDTs are important mycotoxins that provoke potent neurotoxic and tremorgenic symptoms in insects and mammals, at least partly due to their inhibition of potassium ion channels in the nervous system (Dowd et al. 1988; Uhlig et al. 2009; Imlach et al. 2011). In their native ecological contexts, IDTs defend the overwintering structures of the producing fungi, and serve as effector molecules for mutualistic interactions between these fungi and their plant hosts by deterring grazing by large animals and insects (Panaccione et al. 2006; di Menna et al. 2012; Thom et al. 2014). IDT toxicoses of livestock cause significant economic losses in agriculture (Botha et al. 1996; Cawdell-Smith et al. 2010; Philippe 2016). Conversely, IDTs have been considered for the development of pesticides or plant-protecting antifeedants, to be used as components of integrated pest management systems (Panaccione et al. 2014; Saikkonen et al. 2016). Due to their additional, potent and varied bioactivities, IDTs may also serve in the future as drug lead compounds for the development of human and veterinary medications.

IDTs are biosynthesized from geranylgeranyl diphosphate (GGPP) and an indole moiety that originates from a tryptophan precursor (Laws and Mantle 1989; Byrne et al. 2002). After the cloning of the paxilline biosynthetic gene cluster from *Penicillium paxilli* (Young et al. 2001), the biosynthesis of all the major IDT subgroups were also elucidated from diverse filamentous fungi (Zhang et al. 2004; Young et al. 2006; Motoyama et al. 2012; Nicholson et al. 2015; Kozák et al. 2018). These biosynthetic gene clusters share a common, conserved set of core genes, and are supplemented with additional genes that encode enzymes for various tailoring reactions responsible for the idiosyncratic structural elements of the IDT subgroups and the individual IDT congeners (Zhang et al. 2004; Young et al. 2005; Young et al. 2006; Nicholson et al. 2009). In addition, most IDT biosynthetic enzymes show various levels of substrate and product flexibilities, with even the core set of genes differing in their precise characteristics. Together, these variations create a metabolic grid responsible for the remarkable structural diversity of IDTs produced by fungi.

Our knowledge on the regulation of IDT biosynthesis is still limited. Co-regulation of aflatrem production with sclerotia development has been demonstrated in *A. flavus* (Ehrlich and Mack 2014), while a wide spectrum of environmental conditions including temperature, light, various carbon and nitrogen sources were shown to influence penitrem production in *P. crustosum* (Kalinina et al. 2017). Nevertheless, a deeper understanding of mycotoxin production, including the regulation of the biosynthesis of tremorgenic IDTs will be crucial to combat the agricultural threats posed by these fungi (Uhlig et al. 2009; Moyano et al. 2010; Lee et al. 2017). Moreover, further studies are necessary to estimate the real dimensions of the risks posed by these fungi and their harmful metabolites to consumers of agricultural products (Moldes-Anaya et al. 2012; Eriksen et al. 2013). In particular, the co-occurrence and potential synergistic effects of IDTs with other mycotoxins in foods and

feeds warrants further investigations (EFSA Panel on Contaminants in the Food Chain (CONTAM) 2012).

Gaining a deeper insight into the evolution, organization and transcriptional regulation of IDT biosynthetic gene clusters may also provide us with valuable tools to control or even eliminate IDT production during industrial fermentations with fungal species such as *Claviceps paspali*, where the presence of these metabolites represents a safety risk (Kozák et al. 2018). Intriguingly, some paspaline-derived IDTs such as penitrem A may also be regarded as Janus-faced compounds with potential applications in anticancer chemotherapies, either as monotherapies or in combination with other antiproliferative drugs (Sallam et al. 2013a; Sallam et al. 2013b; Goda et al. 2018). Therefore, future metabolic engineering and fermentation optimization studies may need to target the improvement of the yields of these compounds under industrial fermentation conditions (Motoyama et al. 2012; Kalinina et al. 2017). The construction of various fungal synthetic biology platforms for the heterologous production of IDTs with potential biomedical significance is also on the agenda (Tagami et al. 2014; Liu et al. 2015; Tang et al. 2015; Oikawa et al. 2016; Liu et al. 2016).

To the best of our knowledge, this is the first comprehensive review that covers the genetic, biochemical, ecological, veterinary, medical and industrial aspects of the production of these important metabolites by fungi.

Detection and structural characterization of IDTs in biological samples

Although the connection between moldy food and some diseases such as ergotism was suspected for centuries, it was only in the 20th century that the occurrence of secondary metabolite mycotoxins in these foods was discovered to be the molecular basis for such illnesses (Uraguchi 1969). Recent developments of analytical techniques make it increasingly straightforward to detect, even in an untargeted fashion, a large variety of mycotoxins such as IDTs in complex matrices such as food and feed.

Paxilline and paspaline, the simplest IDT congeners, were first isolated half a century ago and characterized by elemental analysis and various spectroscopic methods such as infrared (IR), ultraviolet-visible (UV-Vis), and mass spectroscopy (MS) (Fehr and Acklin 1966; Cole et al. 1974). Although the first nuclear magnetic resonance (NMR) spectroscopy results about paspaline were published in 1977, the assignments of the signals had to be refined almost two decades later (Munday-Finch et al. 1996). Finally, the complete structures of these mycotoxins were elucidated in 1980 by X-ray crystallography (Springer and Clardy 1980).

The discovery of novel IDT subclasses was a relatively slow process in the 1980s, mainly because of the lack of versatile analytical techniques. Immunochemical methods like ELISA were considered the most selective and sensitive analytical tools of the era. However, the development of these methods required the very molecule that needed to be analyzed, making these techniques unsuitable for discovery studies. Thin layer chromatography (TLC) served as the most important tool for both the isolation of unknown molecules and the

identification of known IDTs (El-Banna et al. 1987; Sanchis et al. 1988; Scuteri et al. 1992). In addition to TLC, high performance liquid chromatography (HPLC) became increasingly important in this decade for the analysis of organic compounds, including natural product mycotoxins (Maes et al. 1982; Frisvad 1987; Russell et al. 1989). By 1987 a standardized HPLC method was developed that allowed the detection of all the important groups of mycotoxins and many other fungal secondary metabolites, covering 182 compounds (Frisvad and Thrane 1987). However, the utility of HPLC separation combined with fluorescent detection is rather limited for paspaline-derived IDTs, since only the janthitrems exhibit significant fluorescence (Gallagher et al. 1980b; Lauren and Gallagher 1982). The most traditional mass spectrometric (MS) technique, electron ionization (EI) was also applied for the analysis of mycotoxins. However, this technique is best suited for volatile compounds (Fellows et al. 1981). Although X-ray crystallography provides detailed information about the structures of organic molecules, it has only been rarely applied to study IDTs (Gallagher et al. 1980a; Nozawa et al. 1987; Kawai and Nozawa 1989) since it requires special pretreatments and relatively larger amounts of the sample.

NMR spectroscopic techniques have gone through a remarkable development in the last few decades, resulting in applications such as 2D NMR spectroscopy (Aue et al. 1976) and magnetic resonance imaging (MRI) (Lauterbur 1973). Research towards the structural elucidation of novel IDTs benefited largely from the various 2D NMR techniques (De Jesus et al. 1981; Laakso et al. 1992; Wilkins et al. 1992; Belofsky et al. 1995; Munday-Finch et al. 1995). Although examples of HPLC-coupled NMR applications have been described (Sumarah et al. 2005), these methods have not gained widespread acceptance due to the high cost of the instruments and the lower sensitivity of detection as compared to MS.

The most versatile analytical method for the trace analysis of organic substances is mass spectrometry (MS). This is due to the high sensitivity of MS and its ability to provide structural information such as fragmentation patterns recorded in MS/MS experiments, and elemental composition determined via exact molecular mass measurement with high resolution MS instruments (Q-TOF MS, Orbitrap, etc.). Since the advent of liquid-phase MS interfaces, most importantly the electrospray ion sources, MS analysis can readily be coupled with HPLC allowing sensitive and selective analysis of complex mixtures by measuring directly the molecular mass (typically the mass/charge ratio [m/z] of protonated molecules), including those of new compounds (Naik et al. 1995). The capabilities of the liquid chromatography – mass spectrometry (LC-MS) technique are clearly demonstrated by the rapidly increasing number of target bacterial and fungal metabolites that can be analyzed in a single assay (Sulyok et al. 2006; Sulyok et al. 2007; Vishwanath et al. 2009; Malachova et al. 2014). In addition to multicomponent analysis, the most advanced high-resolution MS approaches allow researchers to perform untargeted LC-MS surveys, resulting in a complex dataset containing information about the molecular masses (normal MS spectra) and about the structures (fragmentation and isotopic patterns) represented in the analyte. The collected datasets can be retrospectively examined even years after the actual measurement, without the need to repeat the experiment (Andersen et al. 2016; Renaud and Sumarah 2016).

Biosynthesis of paspaline-derived IDTs in fungi

Biosynthesis of paspaline

Paspaline is the founding member of the paspaline-derived IDTs that feature an angular hexacyclic ring system consisting of a tetracyclic diterpene fused to an indole moiety (Fig. 1). This scaffold is further elaborated by prenylation, oxidation, reduction, cyclization and chlorination in the various members of the group (Saikia et al. 2007; Tagami et al. 2013; Liu et al. 2016). Paspaline-derived IDTs have been classified in various ways, using historic and structural criteria. Parker *et al.* suggested nine subclasses: the penitrems, janthitrems, lolitrems, aflatrems, terpendoles, shearinines, sulpinines, paxilline and the paspaline/paspalinine/paspalitrems group (Parker and Scott 2005). Another classification by Sings *et al.* divides IDTs into six structural groups: paspalanes, aflatremanes, penitremanes, janthitremanes, lolitremanes and nodulisporanes (Sings and Singh 2003). Nodulisporanes are not derived from paspaline, and lack the F ring of the paspaline skeleton, so they are not discussed in this review.

The cyclic diterpene moiety of paspaline is derived from geranylgeranyl diphosphate (GGPP), biosynthesized from farnesyl diphosphate by the GGPP synthase PaxG in *P. paxilli* and its orthologues in other IDT producers (Fig. 1) (Tagami et al. 2013). Deletion of *paxG* results in the complete elimination of the production of the whole spectrum of IDTs in *P. paxilli* (Young et al. 2001; Saikia and Scott 2009). Orthologues of *paxG* were detected in all characterized IDT gene clusters except that of the terpendole K producer *Tolypocladium album* (Motoyama et al. 2012).

The indole group of IDTs most likely derives from indole-3-glycerol phosphate (Fig. 1). Prenylation of C3 of the indole with the concomitant elimination of glyceraldehyde 3-phosphate yields 3-geranylgeranyl indole (GGI), a common intermediate for all IDTs. This reaction is catalyzed by the prenyl transferase PaxC in *P. paxilli* and its orthologues in other fungi. Although the preferred substrate for prenylation is indole-3-glycerol phosphate, tryptophan was also accepted by recombinant PaxC during *in vitro* studies with the purified enzyme (Tagami et al. 2013). Epoxidation of GGI by PaxM, a FAD-dependent monooxygenase (or its orthologues) yields 10(11)-epoxygeranylgeranyl indole. Cyclization of this intermediate by PaxB or its orthologues affords emindole SB. Finally, another epoxidation-cyclization sequence, catalyzed again by the PaxM - PaxB pair and their orthologues in other fungi, yields paspaline through the formation of the F ring of the IDT skeleton (Tagami et al. 2013; Van de Bittner et al. 2018).

Generation of chemical diversity in the paspaline-derived IDT families

IDT biosynthesis diverges after paspaline, with further modifications of this common intermediate by various oxidations, reductions and prenylations leading to the amazing chemical diversity of the various IDT families (Fig. 2). The first divergent reaction is the oxidation of paspaline. On the first branch, TerQ-catalyzed hydroxylation of paspaline at C11 yields terpendole E in *T. album* (Motoyama et al. 2012). This intermediate is further oxidized by TerP to eliminate the pendant methyl group on C12, yielding 13-desoxyterpendole I that features a C11(12) epoxide and a C10 alcohol (Motoyama et al.

2012). The C11(12) epoxide is also present in lolitrem B produced by *Neotyphodium lolii*, suggesting that the TerQ orthologue LtmQ also possesses C11 hydroxylation activity (Gallagher et al. 1981; Philippe 2016). Correspondingly, the terpendole E-like compound lolicine was isolated from the lolitrem producer *N. lolii* (Munday-Finch et al. 1998). Next, TerQ catalyzes another oxidation, this time the hydroxylation of C13 to form terpendole I. This intermediate is *O*-prenylated at C27 by TerF or its orthologues such as LtmF of *N. lolii* or *Epichloë festucae*. Next, oxidative acetal ring formation by the cytochrome P450 TerK or LtmK gives rise to terpendole C. Additional diprenylation at C20 and C21 by LtmE and oxidative ring closure by the cytochrome P450 LtmJ of *N. lolii* provide the lolitremane IDTs in *N. lolii*/*E. festucae* (Young et al. 2006; Saikia et al. 2012).

On the other branch diverging from paspaline (Fig. 2), the cytochrome P450 monooxygenase PaxP and its orthologues catalyze the oxidative elimination of the pendant methyl group connected to C12 and the formation of the C10 ketone to yield 13-desoxypaxilline via the intermediate *β*-PC-M6 (McMillan et al. 2003). From 13-desoxypaxilline, the pathway again bifurcates. PaxQ-catalyzed hydroxylation of C13 in 13-desoxypaxilline yields paxilline *en route* to the pentirem/jantithrem/sulpinine families of IDTs (McMillan et al. 2003; Nicholson et al. 2015). On the other branch, AtmQ of *A. flavus* and its orthologues such as IdtQ of *C. paspali* also hydroxylate C13, but they oxidize C7 as well to form a cyclic acetal with the C27 alcohol to afford paspalinine. The order of the C13 *vs.* C7 oxidations is still unclear and may even be dissimilar in different fungi (Nicholson et al. 2009; Kozák et al. 2018). Thus, during the elucidation of the IDT product spectrum of *C. paspali*, paxilline and paspalinine were both detected in sclerotia extracts together with paspalicine, the 13-desoxy analogue of paspalinine. This indicates that IdtQ can accept 13-desoxypaxilline for either C7 or C13 oxidation in this fungus (Uhlig et al. 2014). On the other hand, paspalicine could not be isolated during the heterologous production of aflatrems using AtmQ of *A. flavus*, suggesting that C13 hydroxylation of 13-desoxypaxilline may precede the formation of the cyclic acetal with that enzyme (Nicholson et al. 2009). In any case, formation of paspalinine opens the way towards the biosynthesis of the aflatrems, paspalitrems and shearinines. Thus, the substrate and product specificities of the TerQ/PaxQ/AtmQ-like and the TerP/PaxP/AtmP-like cytochrome P450 enzymes in different fungi define the F ring architecture of the IDT skeleton, resulting in the terpendole/lolitrem, the penitrem/janthitrem/sulpinine, and the paspalitrem/aflatrem/shearinine subgroups of paspaline-derived IDTs.

Aflatrem, β-aflatrem, and the paspalitrems are the monoprenylated derivatives of paspalinine (Fig. 2). In the case of aflatrems, the dimethylallyl transferase AtmD catalyzes the γ-selective prenylation of paspalinine at the C20 or the C21 positions to afford aflatrem A and β-aflatrem, respectively. Interestingly, AtmD also accepts paxilline and paspaline as substrates (Liu et al. 2013). While paxilline is prenylated at C20 or C21 just as in paspalinine, paspaline is modified at the C21 or C22 positions. For paspalitrems, the IdtF dimethylallyl transferase of *C. paspali* conducts an α-selective prenylation of paspalinine at the C21 or the C20 positions to yield paspalitrem A or paspalitrem C, respectively. Paspalitrem A is then further oxidized at C32 giving rise to paspalitrem B. For shearinines, the JanD dimethylallyl transferase from *P. janthinellum* conducts two consecutive α-selective prenylations at C21 and C22 to yield shearinine K (Nicholson et al. 2015; Liu et al. 2016). This intermediate undergoes a two-step oxidative cyclization sequence catalyzed by

the JanO FAD-dependent oxidase that yields a bicyclic ring system fused to the paspalinine core, affording shearinine A. Finally, an additional hydroxylation conducted by the cytochrome P450 JanJ gives rise to shearinine D.

At the paxilline branch of the biosynthetic pathway (Fig. 2), the biosynthesis of penitrems, the most elaborate paspaline-derived IDTs, requires 17 enzymes in *P. simplicissimum*. Paxilline is first reduced by PtmH at the C10 ketone to yield the corresponding alcohol, followed by α-selective prenylation at C20 by the dimethylallyl transferase PtmD. The prenylated analogue undergoes an acetylation-elimination sequence catalyzed by PtmV and PtmI to yield 20-prenylpenijanthine with a terminal olefin. The order of the PtmH-PtmD-PtmVI reactions seems to be somewhat flexible (Oikawa et al. 2016). Biosynthesis of janthitrems may follow a similar sequence, with the exception that a diprenylase-oxidative cyclase pair orthologous to JanD-JanO in shearinine biosynthesis is involved in the formation of the bicyclic system fused to the paxilline ring A. Indeed, the *P. paxilli* gene cluster contains the JanD and JanO orthologues PaxD and PaxO, respectively. Paxilline is an excellent substrate for recombinant PaxD that produces 21,22-diprenylpaxilline. While PaxD and PaxO are silent (or at least very weakly expressed) under normal fermentation conditions thus leaving the fungus to produce paxilline as its major IDT product, the *P. paxilli* gene cluster may nevertheless encode the biosynthesis of a janthitrem congener (Liu et al. 2016). Similarly, sulpinine biosynthesis may follow a sequence analogous to that of PtmH-PtmD-PtmVI for penitrems, with the relevant dimethylallyl transferase catalyzing a γ-selective prenylation at C22 on ring A.

For penitrems, γ-hydroxylation of the prenyl side chain by PtmO prefaces a head-to-head coupling with dimethylallyl diphosphate during a prenylation-initiated cationic cyclization event catalyzed by the PtmE dimethylallyl transferase. Oxidative ring expansion by the PtmK cytochrome P450 yields the bicyclo[4,2,0]octane system, followed by the formation of the 8-membered oxocane cyclic ether bridging to C17, catalyzed by another cytochrome P450 (PtmU). Epoxide formation at C11(12) by PtmL, chlorination at C22, and hydroxylation of the bicyclooctane by PtmJ finally affords penitrem A (Liu et al. 2015).

It is important to note that many of the IDT biosynthetic enzymes described here display substrate and product flexibility, leading to a significant metabolic crosstalk among the pathways leading to the three main biogenetic subgroups of IDTs. Consequently, many fungi use a single set of enzymes that form a complex biosynthetic grid with considerable plasticity, thus producing multiple paspaline-derived metabolites that belong to more than one IDT chemical families. Similarly, the structural classification of paspaline-derived IDTs is also somewhat arbitrary and depends on the structural elements or scaffold tailoring events that are considered the most important by the authors. For example, janthithremanes are often understood to include the janthitrems (derived from paxilline) and the shearinines (originating from paspalinine).

IDT production in various fungi

To date, more than 50 fungal species have been demonstrated to produce paspaline-derived IDTs (Supplementary Table S1). The great majority of IDT producer fungi belong to only two ascomycetous classes in the ***Pezizomycotina*** subphylum: the *Eurotiomycetes*

(*Aspergillus*, *Penicillium*, *Emericella* and *Eupenicillium* species in the *Aspergillaceae* and *Trichocomaceae* families within the *Eurotiales* order) and the *Sordariomycetes* (*Claviceps*, *Epichloë*, *Escovopsis, Neotyphodium*, *Periglandula* and *Tolypocladium* species in the *Clavicipitaceae*, *Hypocreaceae* and *Ophiocordycipitaceae* families in the *Hypocreales* order).

In the order *Eurotiales* (class *Eurotiomycetes*), at least 24 *Penicillium* and *Eupenicillium* species have been shown to produce various paspaline-derived IDTs (Supplementary Table S1). The most common IDT in these fungi is penitrem A, a metabolite that is of paramount importance for the contamination of foodstuffs with mycotoxins. One of the most important model organisms used in IDT biosynthetic studies is the saprophytic species *P. paxilli*, which produces paxilline. A set of bioactive paxilline analogues were also isolated from *Penicillium camemberti*, a white mold that is widely used in cheese ripening.

Among the more than 10 *Aspergillus* and *Emericella* species that produce paspaline-derived IDTs, the notorious aflatoxin producer *A. flavus* is also capable of synthetizing aflatrem A and at least two additional aflatrem congeners. Aflatrem and paspaline were also detected in cultures of two other *Aspergillus* species, *A. minisclerotigenes* and *A. parvisclerotigenus.* Importantly, the *koji* mold *Aspergillus. oryzae* was shown to produce 13-desoxypaxilline, a common intermediate for aflatrem biosynthesis. Some other Aspergilli, including *A. desertorum, A. foveolatus* and *A. striatus* are verified paxilline producers while *A. alliaceus* synthesizes two paxilline-like IDTs in axenic cultures.

In the order *Hypocreales* (class *Sordariomycetes*), at least 13 species produce paspaline-derived IDTs (Supplementary Table S1). In the family *Clavicipitaceae*, *C. paspali* and *C. cynodontis* yield paspalitrems while the well-known ergot alkaloid producer *C. purpurea* synthesizes only less complex IDTs such as paspaline. *Epichloë gansuensis* produces paxilline, while the IDT spectra of two additional clavicipitaceous fungi, *N. lolii* and *E. festucae* are rather different. These two fungi form IDTs with the C11(12) epoxide, giving rise to terpendoles and the more elaborate lolitrems. Meanwhile, *Periglandula ipomoeae* strains, symbionts of morning glory, also produce terpendole analogues such as terpendoles C, K, and E (Schardl et al. 2013; Lee et al. 2017; Gardner et al. 2018). Fungi in two other hypocrealean families also produce paspaline-derived IDTs. Thus, *T. album* (basionym *Chaunopycnis alba*; family *Ophiocordycipitaceae*) is another important terpendole producer, while *Escovopsis weberi* (family *Hypocreaceae*) produces shearinines.

Interestingly, the zygomycetous fungus *Mucor irregularis* has recently been shown to yield penitrems and the related rhizovarins. *M. irregularis* (family ***Mucoraceae***, order *Mucorales*, phylum *Mucoromycota*) is the only currently known fungus outside the *Ascomycota* phylum that produces paspaline-derived IDTs (Gao et al. 2016).

Gene clusters for IDT biosynthesis

To date, ten gene clusters for the biosynthesis of the paspaline-derived IDTs have been identified (Fig. 3), including those for penitrems (*P. crustosum* and *P. simplicissimum*) (Liu et al. 2015; Nicholson et al. 2015), paxilline (*P. paxilli*) (Scott et al. 2013), shearinines (*P. janthinellum*) (Nicholson et al. 2015), aflatrems (*A. flavus* and *A. oryzae*) (Zhang et al. 2004; Nicholson et al. 2009), paspalitrems (*C. paspali*) (Kozák et al. 2018), terpendoles (*T.*

album) (Motoyama et al. 2012), and lolitrems (*N. lolli* and its anamorph *E. festucae*) (Young et al. 2006; Saikia et al. 2012). All these gene clusters contain orthologues of the *paxG, paxM, paxC* and *paxB* genes whose enzyme products are collectively responsible for the biosynthesis of paspaline as described in section 3.1 (Fig. 1, Supplementary Table S2). The only exception is the terpendole K gene cluster of *C. alba* that is missing a GGPP synthase gene dedicated to IDT biosynthesis, indicating that this precursor is supplied by the primary metabolic GGPP synthase of the strain (Motoyama et al. 2012).

In addition to the *paxGMCB* orthologues, all biosynthetic gene clusters for the paspaline-derived IDTs elucidated so far also contain genes similar to *paxP* and *paxQ* (Supplementary Table S2). The encoded cytochrome P450 monooxygenases are responsible for the oxidations that channel paspaline towards the paxilline, paspalinine or terpendole E-type cores of the various paspaline-derived IDT families (Fig. 2). All biosynthetic gene clusters for paspaline-derived IDTs also feature additional genes encoding enzymes for the subsequent modification of the angled hexacyclic skeleton (Fig. 3, Supplementary Table S2).

The known biosynthetic gene clusters for the different paspaline-derived IDT families show little synteny. The only exception to date is the pair of clusters for paxilline and the shearinines (Fig. 3), where the order and orientation of the genes are the same, except for the absence of a *janJ* orthologue in the paxilline gene cluster (Liu et al. 2016). In contrast, clusters that are derived from closely related species that produce metabolites belonging to the same IDT family are highly syntenic. For example, *A. flavus* and *A. oryzae* RIB40 contain aflatrem-type gene clusters with identical organizations and chromosomal locations, with the corresponding Atm enzymes of the two species showing at least 95% pairwise identities. Nevertheless, *A. oryzae* produces non-prenylated paspalenes due to a single nucleotide insertion into exon 7 that renders *atmQ* nonfunctional (Nicholson et al. 2009; Qiao et al. 2010; Rank et al. 2012).

Unusually for fungal secondary metabolite biosynthetic gene clusters, several of the IDT clusters seem to be distributed into multiple loci, although sequence closure of draft genomes may lead to the revision of this notion in some cases. Thus, the penitrem biosynthetic genes of *P. simplicissimum*, but not those of *P. crustosum*, are separated into two subclusters. The *ptm2* and *ptm1* subclusters of *P. simplicissimum* are syntenic with genes *PC-23* - *penO* and *penG* - *PC-05*, respectively, within the penitrem cluster of *P. crustosum*. The aflatrem biosynthetic gene clusters of *A. flavus* and *A. oryzae* RIB40 are divided into two loci that reside on different chromosomes (the *atm1* subcluster is located on chromosome 5, while *atm2* is on chromosome 7) (Nicholson et al. 2009). The paspalitrem gene cluster also spans two separate contigs on the genome sequence assembly of *C. paspali* RRC-4128 (Schardl et al. 2013). These two contigs contain all the genes necessary for the biosynthesis of paspalitrems, except for the one that catalyzes C32 hydroxylation of paspalitrem A to yield the end product paspalitrem B (Kozák et al. 2018). Finally, the 11 known genes for lolitrem biosynthesis are dispersed into three *ltm* subclusters in *N. lolli* and *E. festucae*. Subcluster 1 is separated from subcluster 2 by a 35-kb genomic region, while subcluster 3 is located at 16 kb from subcluster 2. The interspersed genomic regions are rich in AT repeats and retrotransposon elements, indicating that the composite *ltm1–3* cluster resides in a rapidly evolving region of the genome, such as a chromosomal sub-telomeric

region. This capacity for rapid evolution is underlined by the observation that the *ltm1–3* composite cluster may have been duplicated in its entirety in *N. lolii* strain Lp19, while subcluster 3 could not be detected in the very closely related strain Lp1. In contrast, *E. festuceae* F11 contains a single copy of the *ltm1*-3 composite cluster (Young et al. 2006).

Regulation of IDT production

The regulation of IDT production has received surprisingly little attention up till now. Thus, no cluster-specific regulator has been validated in any IDT gene cluster so far. Although the penitrem gene clusters of *P. simplicissimum and P. crustosum* feature putative regulatory genes (*ptmS* and PC-06), the function of these genes has not been determined experimentally (Liu et al. 2015). Similarly, the integration of IDT biosynthesis with other metabolic and morphogenetic processes also remains opaque. In *A flavus*, abundant aflatrem production is associated with sclerotia formation (Ehrlich and Mack 2014) while reduced aflatrem production results from the deletion of the *ndsC* gene encoding a global regulator of secondary metabolism and asexual development (Gilbert et al. 2016).

While *N. lolii* and *E. festucae* produce lolitrems *in planta* only (Young et al. 2006), other IDT producers also biosynthesize these compounds in axenic cultures (Motoyama et al. 2012; Nicholson et al. 2015; Kozák et al. 2018). *A. oryzae* produces the paxilline precursor 13-desoxypaxilline under specific growth conditions only (Rank et al. 2012; Fountain et al. 2016). In contrast, *T. album* produces large amounts of terpendoles in various media, suggesting that this biosynthetic pathway is not subject to a strict regulation in this strain (Motoyama et al. 2012). Similarly, *P. crustosum* strains isolated from different environments and substrates consistently produce penitrems (Frisvad and Filtenborg 1983; Yamaguchi et al. 1993; Sonjak et al. 2005). However, a variety of abiotic factors were still found to influence the production of these IDTs (Kalinina et al. 2017). Thus, cultivation in the dark at relatively low temperatures (22 °C); glucose or rhamnose as the carbon source; and supplementation of the medium with glutamate all increase penitrem production by *P. crustosum*. Interestingly, supplementation with tryptophan has the opposite effect in this fungus (Kalinina et al. 2017), in agreement with the notion that indole-3-glycerol phosphate and not tryptophan is the real precursor for IDT biosynthesis (Liu et al. 2015). In contrast, tryptophan serves as both a precursor and an inducer for ergot alkaloid biosynthesis in *C. purpurea* (Řeháček et al. 1971). In *P. nigricans*, penitrem production and sporulation are both induced by calcium chloride (Mantle et al. 1984), while $CuSO_4$ increases penitrem production by *P. crustosum* (Kalinina et al. 2017).

Oxidative stress can also be an important factor for the regulation of tremorgenic IDT biosynthesis. Aflatrem biosynthesis is generally up-regulated in *A. flavus* by hydrogen peroxide, although different isolates react differently to varied concentrations of H_2O_2 (Fountain et al. 2016). In contrast, H_2O_2 has a strong inhibitory effect on penitrem biosynthesis by *P. crustosum.*

IDTs as threats to agriculture, public health, and the fermentation industries

Agricultural threats

Among the known IDTs, lolitrems and paspalitrems represent the most severe danger for livestock. These compounds cause a tremorgenic syndrome in grazing animals, referred to as “ryegrass stagger” in the case of lolitrem B ingestion (Fletcher and Harvey 1981; Gallagher et al. 1981), and “Paspalum stagger” or “Bermuda grass stagger” in the case of paspalitrem mycotoxicoses (Cole et al. 1977; Uhlig et al. 2009). Ryegrass stagger is typically caused by grazing on *Lolium perenne* (perennial ryegrass) infected by *N. lolii* (Gallagher et al. 1981), since lolitrem B is abundant in the *N. lolii* - *L. perenne* association (Philippe 2016). Ryegrass stagger is most frequently reported in New Zealand and Australia, and the affected animals include sheep, cattle and horses (di Menna et al. 2012). To manage ryegrass stagger, different endophyte strains with altered mycotoxin production spectra were isolated and tested. Amongst these, the endophytic fungus AR37 does not produce lolitrem B, but biosynthesizes epoxy-janthitrem in high concentrations. While epoxy-janthitrem is just as toxic to insects as lolitrem B, it does not cause a tremorgenic syndrome on grazing animals (Thom et al. 2013).

Outbreaks of Paspalum stagger is frequent in South Africa but case reports from the Americas, Europe, and New Zealand were also published (Mantle et al. 1978; Moyano et al. 2010; Cawdell-Smith et al. 2010). *C. paspali* infecting *Paspalum dilatatum* (Dallis grass) and *C. cynodontis* infecting *Cynodon dactylon* (Bermuda grass) produce similar paspalitrem IDT profiles (Uhlig et al. 2009). Ingestion of sclerotia containing these toxins causes a tremorgenic syndrome with very similar symptoms to that of ryegrass stagger (Moyano et al. 2010). Just as with lolitrem B intoxication, the affected animals usually recover rapidly after being removed from the infected pasture (Moyano et al. 2010; Cawdell-Smith et al. 2010).

Grazing on morning glories, most frequently on *Ipomoea asarifolia* and *Ipomoea muelleri*, may also cause a tremorgenic syndrome on livestock (Gardiner et al. 1965; Medeiros et al. 2003; Dorling et al. 2004; Carvalho de Lucena et al. 2014). This toxicosis is also associated with the presence of IDTs (Lee et al. 2017), produced by seed-transmitted endophytic fungi, most likely *P. ipomoeae* (Schardl et al. 2013; Lee et al. 2017). The main IDT congeners isolated from endophyte-infected *I. asarifolia* and *I. muelleri* are terpendole K, 11-hydroxy-12,13-epoxyterpendole K and 6,7-dehydroterpendole A (Lee et al. 2017).

A ryegrass stagger-like syndrome, “huecu’s disease” has been observed on sheep, horses, cattle and goats in Argentina. This is caused by the ingestion of the grass *Poa huecu* contaminated with penitrem A and B, produced by *Penicillium* species (Scuteri et al. 1992). Penitrem A may also contaminate the soil, and ingestion of such soil by grazing animals can also cause a tremorgenic syndrome (Patterson et al. 1979). However, most case reports of penitrem A intoxications involve the ingestion of moldy food by pets, most frequently dogs (Hayes et al. 1976; Richard et al. 1981; Hocking et al. 1988; Walter 2002). Symptoms on dogs include generalized convulsion, ataxia, vomiting, tremors, frequent urination and defecation, mydriasis (dilation of the pupil), polypnea (panting), and hyperthermia (Richard

and Arp 1979; Richard et al. 1981). Typically, the affected animals recover within a few weeks or months, but ataxia may still remain observable even years later in severe cases of intoxication (Eriksen et al. 2010).

Contamination of animal feedstuffs by spoilage fungi that produce mycotoxins, including IDTs such as penitrem A (Stoev et al. 2010), represents another worldwide threat to the livestock industry. Such problems are independent of the geographical location or the origin of the feed from industrial or family-owned farms (EFSA Panel on Contaminants in the Food Chain (CONTAM) 2012).

Public health threats

Amongst IDTs, the toxic effects of only penitrem A and its analogues have been studied in depth, mainly in mice (Eriksen et al. 2013). Valuable information on the adverse physiological effects of these compounds on mammals also came from case reports on poisoned dogs that consumed various moldy foods infected by *P. crustosum* (Eriksen et al. 2010; Eriksen et al. 2013). Tremorgenic mycotoxicoses likely caused by penitrem A and/or other mycotoxins produced *P. crustosum* have only been reported very rarely in humans (Eriksen et al. 2013), and were connected to either the consumption of mold-contaminated food (Lewis et al. 2005) or drink (Cole et al. 1983), or resulted from exposure to moldy silage (Gordon 1993). It is important to note that food wastes from private households may contain high concentrations of tremorgenic mycotoxins, *e.g.* as high as 35–7,500 μg/kg of penitrem A (Rundberget et al. 2004)

The neurotoxic effects of penitrem A include tremors, convulsions, ataxia and nystagmus (involuntary eye movement) (Eriksen et al. 2013). In humans, symptoms affecting the gastrointestinal tract, such as nausea, vomiting, and bloody diarrhea have also been recorded (Cole et al. 1983). The toxicokinetic characteristics of this lipophilic molecule are relatively well-known and include (i) rapid absorption through biological membranes, (ii) rapid distribution within the body through the blood vessels to the liver, the kidneys and the central nervous system (penitrem A can penetrate the blood-brain barrier), (iii) metabolism in the liver to yield more hydrophilic compounds and (iv) excretion through the bile into the feces (Moldes-Anaya et al. 2009; Eriksen et al. 2010; Moldes-Anaya et al. 2012; Eriksen et al. 2013).

Penitrem A interferes with GABAergic neurotransmission in the central nervous system by inhibiting the $GABA_{(A)}$ receptors in the forebrain and in the cerebellum in a region-specific manner (Moldes-Anaya et al. 2011; Eriksen et al. 2013). In addition, this IDT is a potent antagonist of the high-conductance Ca^{2+}-activated potassium channels (BK channels) in smooth muscles and peripheral tissues (Knaus et al. 1994; Eriksen et al. 2013). Importantly, lolitrem B and paxilline produced by *N. lolii* and *E. festuce* also inhibit these potassium channels, leading to ryegrass staggers in livestock (Dalziel et al. 2005; Imlach et al. 2008). Penitrem A-induced neurotoxicity may also be linked to the oxidative stress-inducing effect of this compound, as shown in cerebellar granule neurons in rats (Berntsen et al. 2017).

Additional harmful physiological effects of IDTs may include genotoxicity, as observed for paxillin in human lymphocytes (Sabater-Vilar et al. 2003). It is noteworthy that both *P.*

crustosum extracts and penitrem A showed considerable cytotoxicity *in vitro* against human lung cancer, human hepatoma carcinoma, murine fibroblast and murine neuroblastoma cell lines (Bunger 2004). The physiological effects of IDTs might be even more varied and severe because toxigenic fungi typically produce a wide spectrum of harmful secondary metabolites (Bunger 2004; Andersen and Frisvad 2004; Santini et al. 2014; Prencipe et al. 2018). Unfortunately, the synergistic behavior of these compounds is a notoriously understudied area of mycotoxicology.

Paspalitrem IDTs are relatively rarely implicated in outbreaks of toxic syndromes in humans. One such case involved an outbreak of tremors in India in 1946. At that time, *Paspalum scrobiculatum*, a type of millet, was consumed in certain parts of India because of a shortage of rice. Tellingly, the paspalitrem producer *C. paspali* was isolated from the unwholesome grain (Aaronson 1988). Even today, another IDT producer, *C. purpurea* is often isolated from rye and barley, and paxilline can be detected in such specimen at the maximum concentration of 0.6 mg/kg (Bauer et al., 2017). *C. purpurea* sclerotia contain at least seven paspalenes, including paspaline (Uhlig et al. 2014). This raises the interesting possibility that these toxins may have played a role in the development of the feared symptoms of ergotism in the Middle Ages. Some authors, including Bauer and coworkers hypothesized that IDTs might have contributed to outbreaks of convulsive ergotism during history (Bauer et al. 2017).

Although the daily exposure of consumers to various IDTs is unknown, these toxic compounds have been detected in a number of food and drink products in highly variable concentrations, depending on the geographical location. Penitrem A and its producer, the food spoilage fungus *P. crustosum* have been detected in several agricultural products and foodstuff, such as beer, cheese, chestnut, meat products, vegetables, pudding, grape berries, honey, sausage, etc. (El-Banna and Leistner 1988; Overy et al. 2003; Sengun et al. 2008; Tancinova and Labuda 2009; Kacaniova et al. 2012; Santini et al. 2014; Camardo Leggieri et al. 2016; Olsen et al. 2017; Prencipe et al. 2018). Of course, penitrem A is not the only IDT that can be detected in contaminated foods. Paxilline and other IDTs were found in moldy tomatoes infected with *Penicillium tularense* (Andersen and Frisvad 2004). Lolitrem B and epoxy-janthitrem were observed in the fat and milk of growing and lactating animals that grazed on contaminated tall fescue (Miyazaki et al. 2004; Finch et al. 2012; Finch et al. 2013; Shimada et al. 2013; Zbib et al. 2015). The maximum concentration of these IDTs in cow milk reached 5 ng/ml and 109 ng/ml for lolitrem B and epoxy-janthitrem, respectively (Finch et al. 2013). Miyazaki *et al.* (2004) measured 210 ppb lolitrem B in the perirenal fat of Japanese Black cattle feeding on contaminated ryegrass (Miyazaki et al. 2004). However, the concentrations of these IDTs decrease rapidly *in vivo* when the animals are removed from the contaminated pasture, thus reducing the prevalence of food containing these two mycotoxins and limiting the threat to human health (Miyazaki et al. 2004; Finch et al. 2013).

Today, highly sensitive HPLC-MS methods are available to facilitate the detection of mycotoxin contaminants in food and drink samples (Rundberget and Wilkins 2002). A recent survey (Kalinina et al. 2018) revealed that 10% of cheese samples taken from the European single market contained penitrem A at an average concentration of 28.4 μg/kg and with a maximum concentration of 429 μg/kg. Considering such observations, it would be

important to devise standardized analytical protocols for tremorgenic IDT mycotoxins. Even more importantly, it would be highly desirable for national and international regulatory agencies to define the maximum legally permitted levels of tremorgenic IDTs in human food and animal feed, similar to legal limits established for other mycotoxins such as aflatoxin (Medeiros et al. 2012; Oliveira et al. 2014).

Threats for the fermentations industries

C. paspali is used to produce ergot alkaloids in the pharmaceutical industries for the manufacture of drugs against migraine and for the treatment of Parkinson's disease. IDT biosynthesis by *C. paspali* during industrial fermentations or agricultural production on infected grasses represents both an economic and a safety problem for the manufacturers. Precursors and cofactors that may be utilized for ergot production are depleted by IDT biosynthesis, reducing productivity. At the same time, IDTs are hazardous impurities that must be removed from ergot alkaloid products and must also be safely disposed (Kozák et al. 2018).

A. oryzae is widely used in biotechnology and the food industry. This fungus is the domesticated descendant of *A. flavus*. While the genotypes of *A. oryzae* and *A. flavus* are nearly identical, these fungi can still be distinguished by their morphological and physiological characteristics, and by their secondary metabolite profiles (Frisvad et al. 2018). While *A. oryzae* does not produce aflatoxin (Barbesgaard et al. 1992; Tao and Chung 2014), certain isolates of *A. oryzae* were demonstrated to produce tremorgenic paspaline-type IDTs such as 13-desoxypaxilline (Qiao et al. 2010; Rank et al. 2012). Production of these IDTs may represent an overlooked risk factor for fermented food products.

A possible solution may be to eliminate the production of IDT mycotoxins during various fermentations, for example by inactivating key IDT biosynthetic genes. This was demonstrated by the knockout of the *idtCBGF* paspalitrem biosynthetic genes in the industrially important ergot producer *C. paspali*. This led to the complete abrogation of IDT biosynthesis during fermentations, while the production of ergot alkaloids remained undisturbed with this strain (Kozák et al. 2018).

Future perspectives

Potential medical applications

Paspaline-derived IDTs are not in current medical use. Nevertheless, these metabolites show potent and wide-ranging bioactivities and have been considered for pharmaceutical development over the years. It is noteworthy that terpendole congeners were isolated in a systematic screening for acyl-CoA:cholesterol acyltransferase (ACAT) inhibitors of microbial origin (Huang et al. 1995). ACAT inhibitors are potential agents for the prevention of atherosclerosis (Lee et al. 1998). The most potent inhibitor is terpendole C (IC_{50}: 2.1 μM) but terpendole D (IC_{50}: 3.2 μM) was considered even more promising since that congener displayed relatively low cytotoxicity against J774 macrophages (Huang et al. 1995). After the discovery of two ACAT isozymes in mammals (Anderson et al. 1998; Cases et al. 1998), isozyme selectivity became a prerequisite for potential anti-atherosclerotic agents

(Giovannoni et al. 2003). Testing the selectivity of a number of microbial ACAT inhibitors revealed that terpendole C inhibits both ACAT isozymes, reducing the enthusiasm for the further clinical development of these IDTs (Ohshiro et al. 2007).

Terpendoles were re-discovered in a systematic screening for specific M phase cell cycle inhibitors where terpendole E was found to specifically inhibit the human kinesin Eg5 (Nakazawa et al. 2003), a potential target for cancer therapy (Knight and Parrish 2008; Sarli and Giannis 2008). Terpendole E does not affect microtubules directly, but induces the formation of monopolar mitotic spindles in the M phase, similar to monastrol (Nakazawa et al. 2003; Tarui et al. 2014; Sheff et al. 2017). To increase the production of terpendole E that is an intermediate of terpendole K biosynthesis and is thus produced only in low amounts, the *terP* gene was inactivated in *T. album* leading to the accumulation of terpendole E (Motoyama et al. 2012). The same *terP* knockout strain also produces the novel shunt product 11-ketopaspaline (Tarui et al. 2014). Later studies revealed that terpendole E and 11-ketopaspaline are both potent inhibitors of the microtubule-stimulated ATPase activity of Eg5. Importantly, these terpendoles not only inhibit the wild type Eg5, but retain excellent activity against Eg5 variants that are resistant to the known Eg5 inhibitors *S*-trityl-L-cysteine and GSK-1 (Tarui et al. 2014).

Penitrems show *in vitro* antiproliferative, anti-haptotactic (cell migration inhibitory) and anti-invasive activities against human breast cancer cell lines. Penitrem A induces G1 cell cycle arrest and up-regulates the arrest protein p27 (Goda et al. 2018). The documented synergistic effects of penitrem A treatment with anti-HER drugs may have a significant impact on future development of breast cancer chemotherapies (Goda et al. 2018). Interestingly, the early biosynthetic intermediates paspaline and emindole SB also show noticeable antiproliferative and antimigratory activities, with the anti-haptotactic activity of paspaline almost equipotent to that of the more elaborate congener penitrem A (Sallam et al. 2013a; Sallam et al. 2013b). These notable activities of penitrem A are due, at least in part, to the inhibition of the Wnt/β-catenin pathway (Sallam et al. 2013a) that is a validated target of novel anticancer drugs (Lu et al. 2011). However, the BK channel (high-conductance Ca^{2+}-activated potassium channel) inhibitory and tremorgenic activities of these IDTs present a formidable obstacle towards the development of penitrems as novel drugs (Sings and Singh 2003). Inhibition of the α-subunit of the BK channel interferes with neurotransmitter release mechanisms and neuroreceptors in the central and the peripheric nervous systems. To overcome these challenges, new penitrem analogues were synthesized. Some of these lack the BK channel inhibitory and tremorgenic activities while still repress β-catenin in human breast cancer cells (Sallam et al. 2013a). Another interesting approach is to mitigate the undesired effects of penitrems in a combination therapy with preventative agents such as astaxanthin or docosahexaenoic acid. Simultaneous application of penitrem A with these agents in rats significantly reduced toxicity and reversed the BK channel blockade associated with penitrem A alone (Goda et al. 2016).

While paspaline-derived IDTs are associated with tremorgenic activities, emindole SB-derived IDTs such as nodulisporic acids display highly potent insecticidal activities without observable adverse effects in mammals (Shoop et al. 2001). Thus, non-tremorgenic IDT

analogues may find applications as pesticides in agriculture or even as anti-parasitic agents against insects feeding on humans.

Conversely, the potent BK channel inhibitory activity of penitrems may also be utilized in the future. The standard inhibitor for BK channels is iberiotoxin whose high price and membrane impermeability makes its use less than ideal for studies in organ models or whole animals. The higher membrane permeability, potency and efficacy of penitrem A may recommend this IDT as a good alternative to iberiotoxin for studying BK channels *in vitro* and *in vivo* (Stewart et al. 2012; Asano et al. 2012; Kyle et al. 2013; Needham et al. 2014).

Invasive fungal infections are an important medical problem, particularly in immunocompromised patients. However, treatment of invasive candidiasis is restricted to only a few drug families with a limited number of mechanisms of action (Tkacz and DiDomenico 2001). The toxicity of amphotericin B, and the emergence of resistance in the clinically relevant *Candida albicans* species against the azoles and the candins presents a clear demand for the development of new therapeutic strategies (Kathiravan et al. 2012). Importantly, shearinines D and E block the formation of biofilms by *C. albicans* (You et al. 2013). Biofilm formation makes the treatment of fungal infections very problematic, because biofilms constitute a barrier that can prevent antifungal drugs from reaching the fungal cells (Douglas 2003; Nett et al. 2010; Nett et al. 2011). *C. albicans* treated with shearinine D forms an irregular, sparse layer instead of a well-developed biofilm. Co-application of shearinine D or E synergistically enhanced the potency of amphotericin B against clinical *Candida* isolates (You et al. 2013).

Systematic screening for natural compounds active against the influenza A virus subtype H1N1 led to the isolation and characterization of an array of IDT congeners from *P. camemberti*. Three novel and six known paspaline- and paxilline-derived IDT analogues showed significant antiviral activity (IC_{50}: 17.7 – 73.3 μM) (Fan et al. 2013), raising hopes that novel antiviral agents may be developed from these IDTs in the future.

Industrial scale production of IDTs using filamentous fungi

The industrial-scale production of paspaline-derived IDTs for future medical and agricultural applications requires careful consideration of the potential producer strains. For example, *P. paxilli* has been extensively used to clarify the individual steps of IDT production (Young et al. 2001). However, the pharmaceutical industry has yet to invest in the strain development and fermentation process optimization of this strain. Similarly, *N. lolii* may be difficult to adopt for the large-scale production of secondary metabolites due to its fastidious growth habits, genetic instability, and its requirement for a plant host for IDT production (Wiewióra et al. 2015).

Only a few studies investigated the production of IDTs in laboratory scale fermentations. Thus, *P. nigricans* produced 60 mg/L penitrem in a 60 L stirred fermentor in 5 days (Mantle et al. 1984). Engineered *C. alba* accumulated 36 mg/L terpendole E, an intermediate of terpendole K biosynthesis, in a 30 L fermentor in 2 days (Motoyama et al. 2012). Kalinina and coworkers demonstrated that the spectrum of penitrems produced by *P. crustosum* can also be controlled by varying the chemical and physical conditions of the fermentation

(Kalinina et al. 2017). Encouragingly, *C. paspali* is currently used for ergot alkaloid production in the pharmaceutical industry. Although these fermentations have not been optimized for IDT production, the significant knowledge base available for the safe and economical industrial scale fermentation and the genetic manipulation of this fungus (Arcamone et al. 1960; Tudzynski et al. 2001; Kozák et al. 2018) may recommend *C. paspali* as a useful candidate to produce IDT congeners in the future.

Heterologous biosynthesis of IDTs

Domesticated microbial hosts for the heterologous expression of biosynthetic pathways are becoming increasingly important for the characterization of biosynthetic pathways and the clarification of reaction mechanisms. As a prominent example, the Oikawa group has been developing a heterologous production system for secondary metabolites. In this system, biosynthetic genes are cloned into multiple expression vectors and co-integrated into the genome of the domesticated filamentous fungus *A. oryzae*. In an influential set of publications, Oikawa and coworkers have used this expression system for the reconstitution of the biosynthesis of various IDTs to clarify the individual biosynthetic steps (Oikawa et al. 2016). Thus, the biosynthesis of paspaline was reconstituted in 2012 using *A. oryzae* transformants producing the *P. paxilli* IDT biosynthetic enzymes PaxG, PaxC, PaxM and PaxB (Liu et al. 2015). Adding the *paxP* and *paxQ* genes yielded paxilline in *A. oryzae*. In 2014, seven genes from the aflatrem biosynthetic locus of *A. flavus* were expressed in the *A. oryzae* host, leading to the heterologous production of aflatrem A and β-aflatrem in addition to paspaline and paspalinine (Tagami et al. 2014). In 2015, the same *A. oryzae* chassis was utilized to dissect the biosynthesis of penitrems by functionally analyzing 13 of the 17 genes encoded in the biosynthetic gene cluster of *P. simplicissimum* (Liu et al. 2015). The same *A. oryzae* heterologous expression system was also used to clarify the biosynthesis of shearinines from *P. janthinellum* (Liu et al. 2016).

Other hosts have also been used to express IDT biosynthetic gene clusters. For example, a versatile multigene expression system termed MIDAS was developed to express biosynthetic gene clusters in a *P. paxilli* strain with the entire paxilline biosynthetic cluster deleted (van Dolleweerd et al. 2018). This system was used to express key biosynthetic genes from *Hypoxylon pulicicidum* to produce nodulisporic acid intermediates and congeners (Van de Bittner et al. 2018). Although nodulisporic acid is not a paspaline-derived IDT and is thus not the subject of this review, the work still shows that strains derived from IDT producers can be useful hosts to produce other bioactive molecules. Meanwhile, nodulisporic acid derivatives are potent insecticides that lack observable adverse effects in mammals, including the tremorgenic activities associated with the paspaline-derived IDT core.

Combinatorial biosynthesis of novel, unnatural IDTs in synthetic biological platforms is another promising application. To design such a platform, the Tang group has used *Saccharomyces cerevisiae* as the chassis for the heterologous production of epoxy-geranylgeranyl indole (Tang et al. 2015). Co-expression of various IDT cyclases, some with previously unknown product specificities yielded not only paspaline (1.5 mg/L), but also various seco-IDTs and aflavinine- or anominine-type Markovnikov-derived cyclic scaffolds. This work demonstrates that diversity-oriented combinatorial biosynthesis with enzymes

coopted from distinct and even orthogonal IDT biosynthetic pathways may be used to generate diverse, drug-like chemical matter towards the discovery of future medications. Presumably, such synthetic biological platforms will also be useful in the future for the large-scale and economical production of IDT congeners for medical or agricultural applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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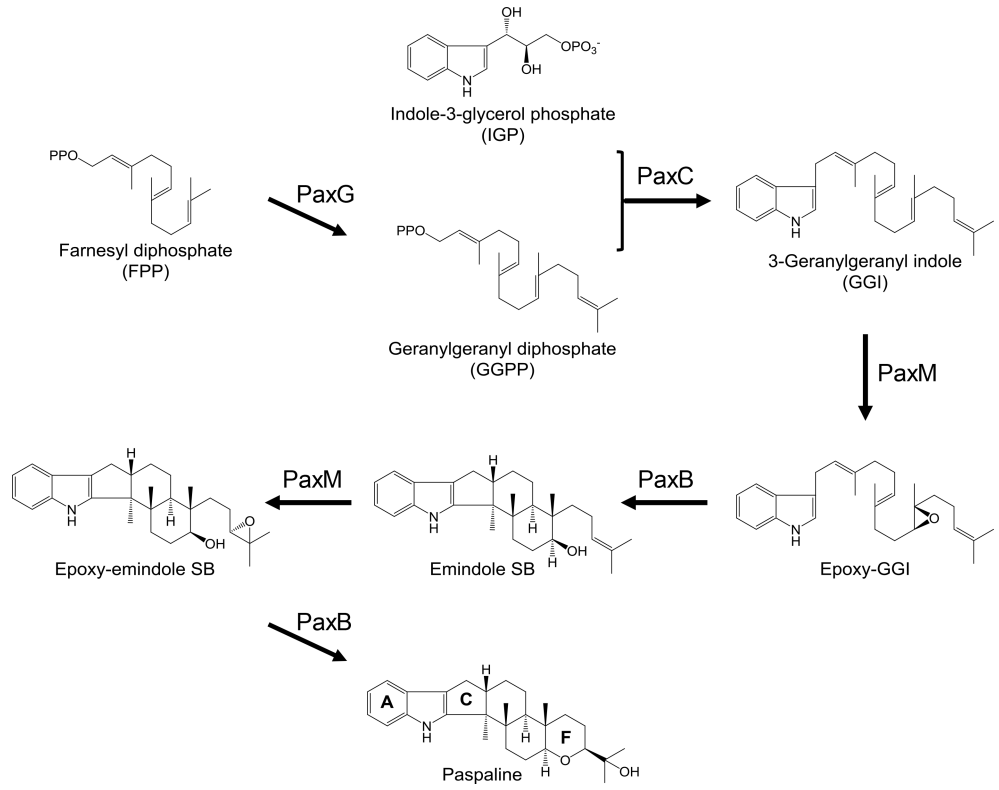

Figure 1. Biosynthesis of paspaline in *Penicillium paxilli*.

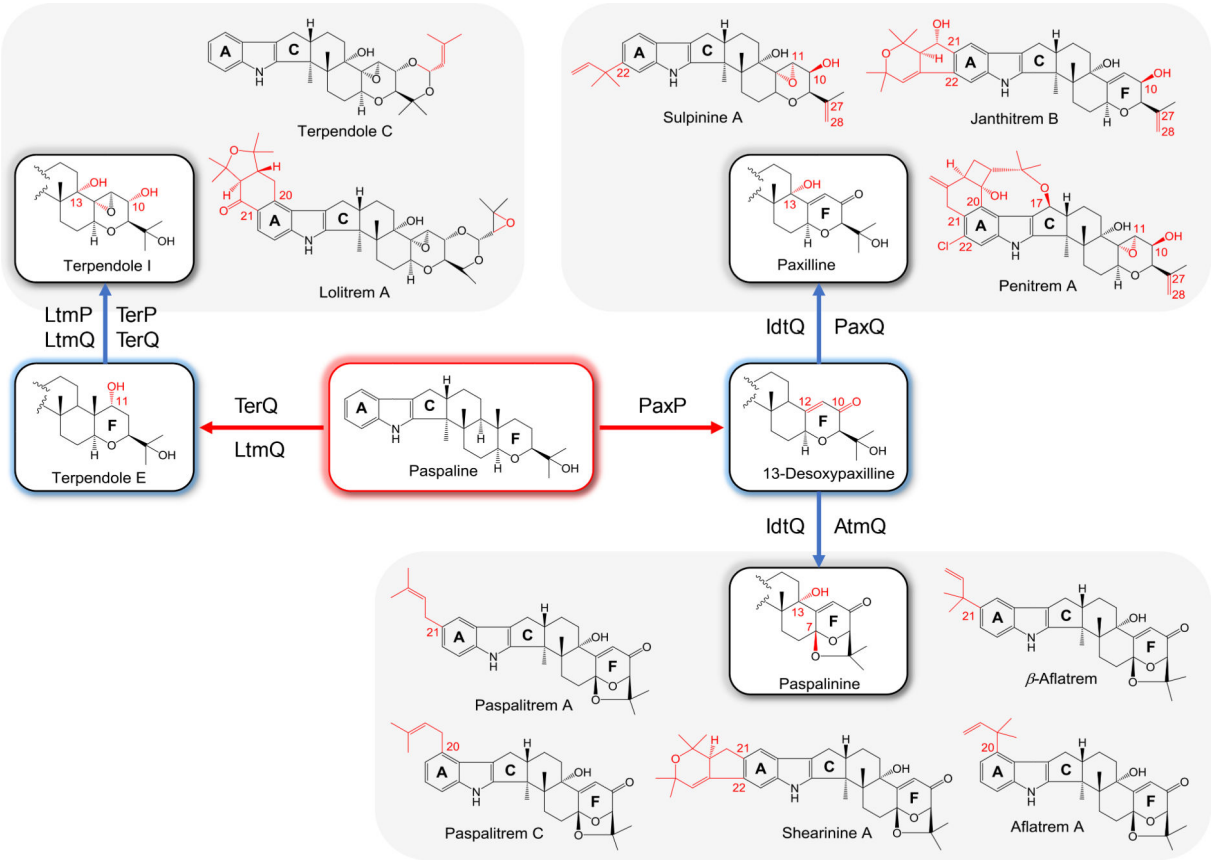

Figure 2. Biosynthesis of the three main subgroups of paspaline-derived indole-diterpenes.
Only the main diversity-generating biosynthetic steps and the corresponding enzymes are shown.

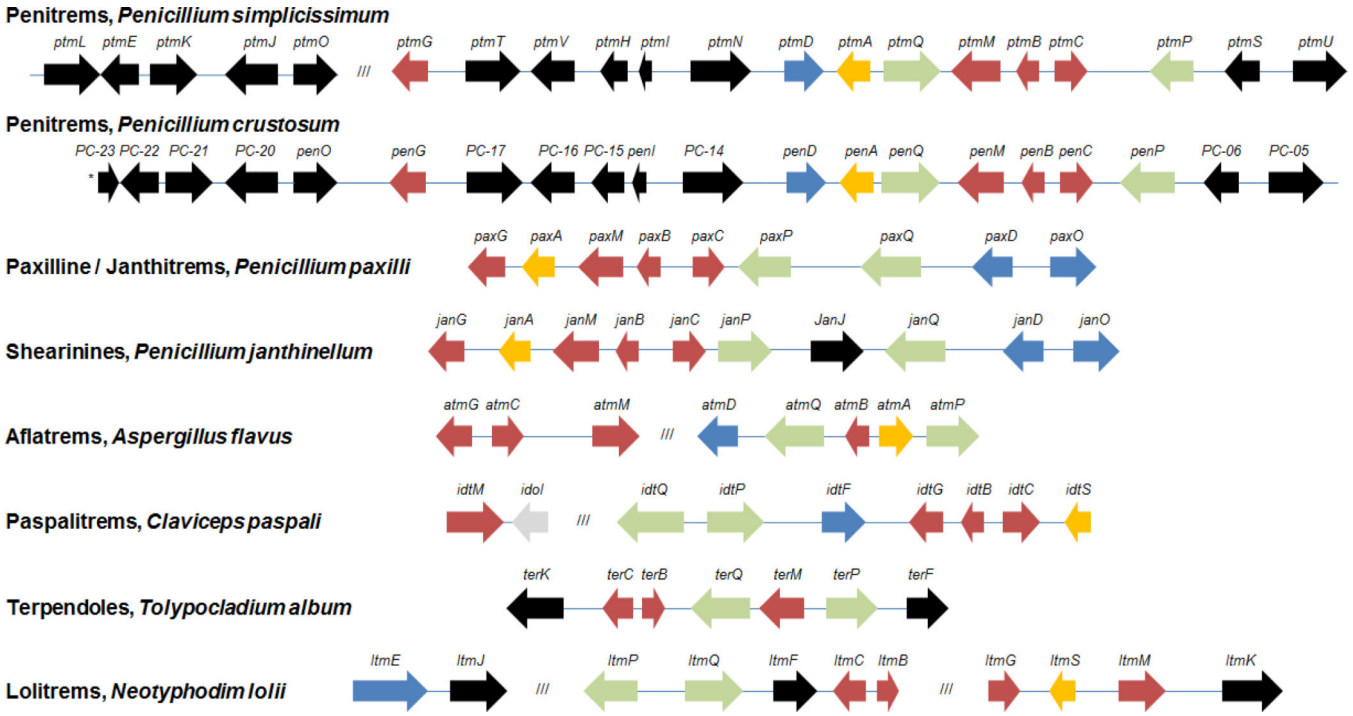

Figure 3. IDT biosynthetic gene clusters.

Genes encoding enzymes for: paspaline biosynthesis (*red*); oxidation of the paspaline rings E and F (*green*); prenylation of C20, C21 or C22 of paspaline (*blue*); unknown membrane-associated processes (*yellow*); and IDT group-specific tailoring reactions (*black*) are highlighted. See Supplementary Table S2 for protein similarities. Practically identical gene clusters for aflatrems in *A. flavus* and *A. oryzae*, and for lolitrems in *N. lolii* and *E. festucae* are represented by only one cluster each. *, End of the contig (*P. crustosum*).